EXHIBIT P

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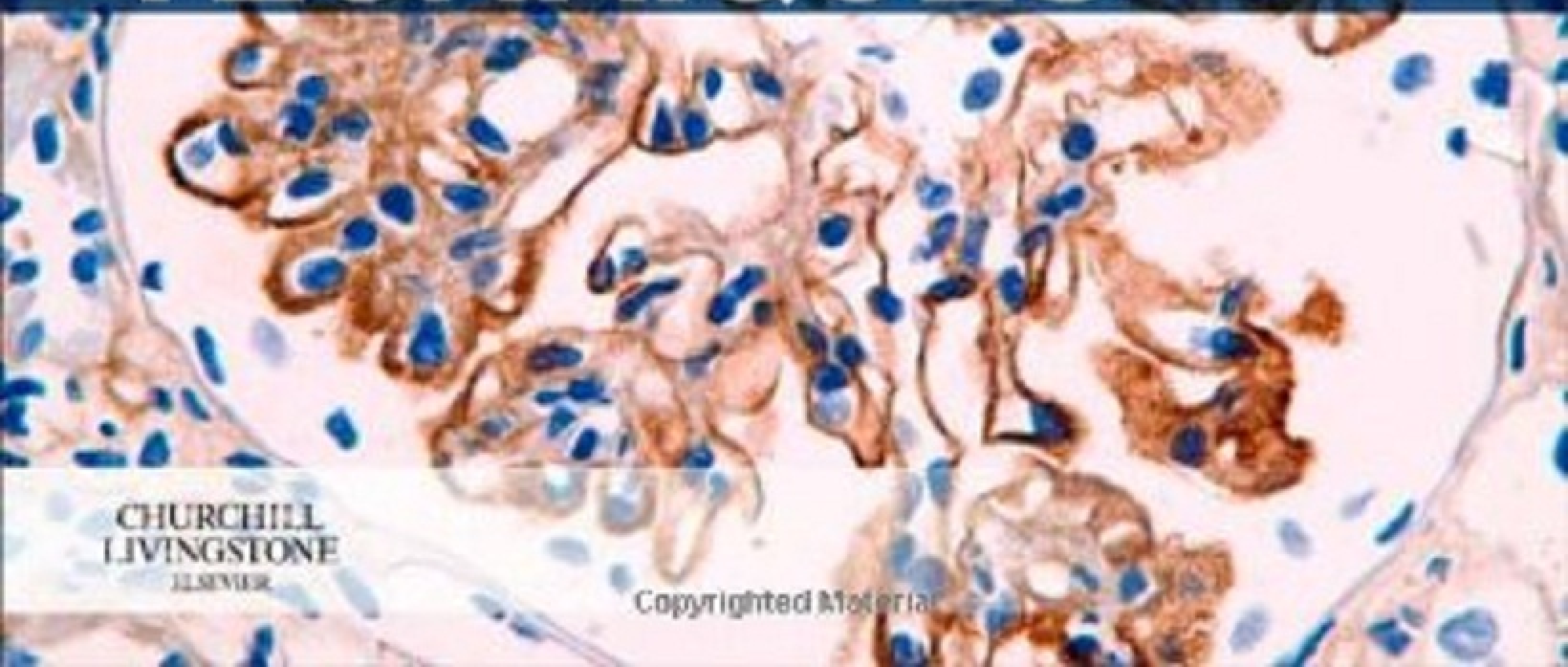
Bancroft's THEORY and PRACTICE of HISTOLOGICAL TECHNIQUES

SEVENTH EDITION



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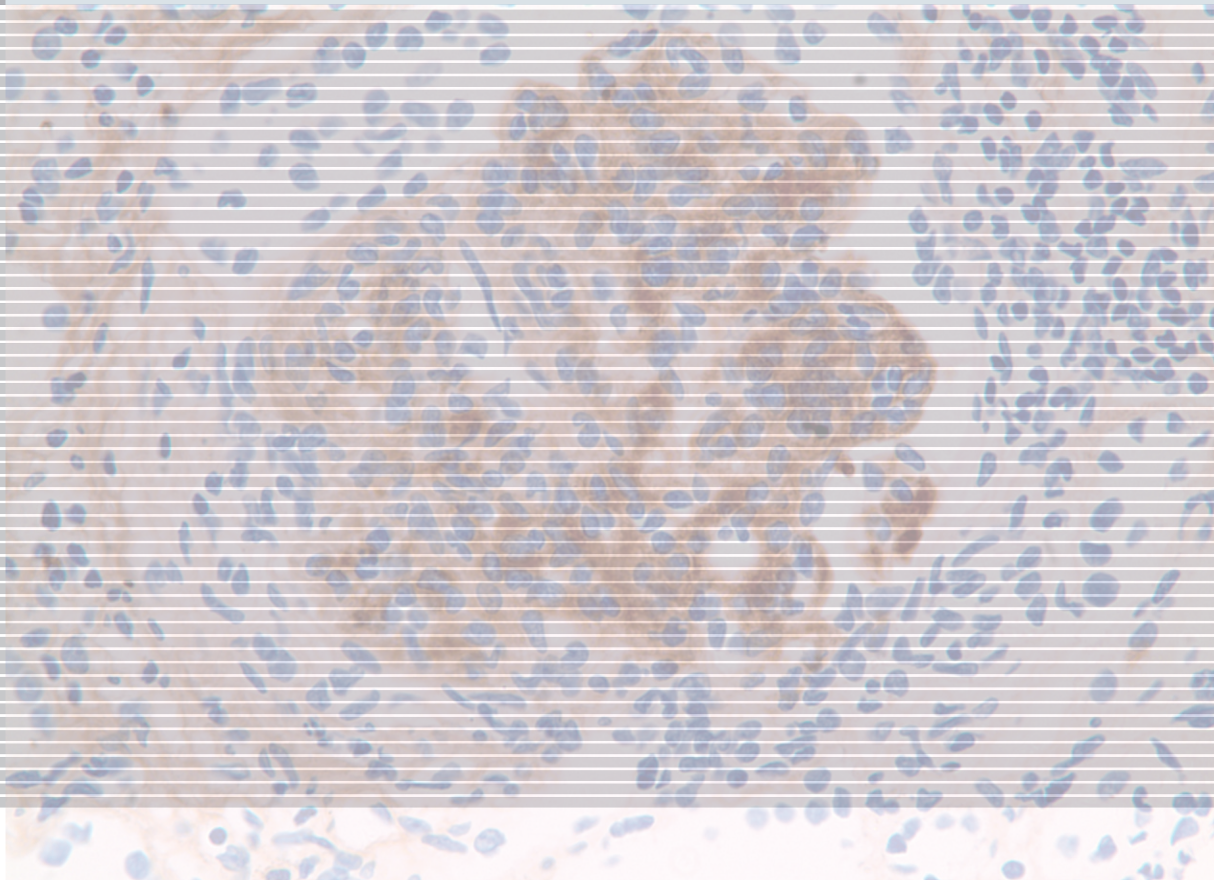
Bancroft's Theory and Practice of Histological Techniques



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Bancroft's THEORY and PRACTICE of HISTOLOGICAL TECHNIQUES

SEVENTH EDITION

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and accredit laboratories according to ISO standards within their own country, national accreditation bodies such as CPA in the UK must themselves be accredited under this standard.

Laboratories wishing to be accredited must demonstrate a robust quality management system and consistent application of the standards, usually by undergoing assessment and surveillance visits from the accrediting body, plus providing annual reviews of the quality management system.

Quality control (QC)

This system checks that the work process is functioning properly. It includes processes utilized in the laboratory to recognize and eliminate errors. It ensures that the quality of work produced by the laboratory conforms to specified requirements prior to its release for diagnosis. Errors and/or deviations from expected results must be documented and include the corrective action taken, if required. In the laboratory, quality control has long been a component of accreditation requirements and should be ingrained in scientists as a daily practice.

Most laboratories have experienced scientists and support staff who have the responsibility of performing routine quality control checks prior to the release of slides for diagnosis. This QC evaluation will include, but is not limited to: accurate patient identification, fixation, adequate processing, appropriate embedding techniques, acceptable microtomy, unacceptable artifacts, and inspection of controls to determine quality and specificity of special staining and immunohistochemistry methods. Criteria should be established that would trigger a repeat if the QC findings were qualitatively or quantitatively unacceptable. Despite having a conscientious QC system in the laboratory, pathologists (having a higher level of expertise) perform the final QC examination as they assess/report the slide. It is their responsibility to determine that this is adequate for diagnostic interpretation. However, all personnel are responsible, such that errors and incidents should be recorded and audited regularly to identify trends. This will highlight any training needs and gaps.

External quality assurance (EQA)

In addition to local data collection and monitoring for internal quality control, external mechanisms provide valuable information regarding quality and peer comparisons and as an educational tool. In the UK, quality assurance of laboratory techniques is organized on a national basis. It is a system of peer review and registration with appropriate (approved) schemes. The non-profit-making NEQAS (National External Quality Assurance Scheme) organizes programs for histochemistry and immunohistochemistry.

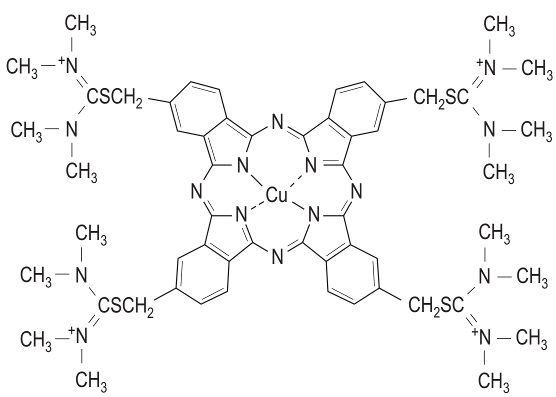
In the USA, the National Society of Histotechnology (NSH) in partnership with the College of American Pathologists (CAP) (2006), created the Histology Quality Improvement Program (HistoQIP). Their system scores each slide, assessing the fixation, processing, embedding, microtomy, staining and coverslipping. Additionally, CAP establishes national surveys for immunohistochemistry.

The UK quality assurance schemes were started by members of the profession to establish quality standards within histopathology. Registration with the schemes is now a requirement for accreditation. The quality assurance process is based on peer review of the stained sections submitted by participating laboratories. There are also medical quality assurance schemes for pathologists that cover many of the sub-specialties of histopathology.

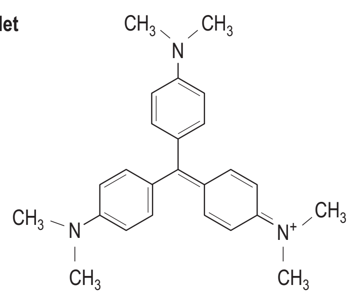
The quality assurance schemes currently used in the UK are coordinated under the auspices of UK NEQAS and within this organization there are two individual schemes for histopathology, the NEQAS for immunohistochemistry and the NEQAS for cellular pathology techniques. The immunohistochemistry scheme gives participants the option to be assessed on general antibody panels, or more specialist laboratories may choose to participate only in the lymphoma or breast specialist areas. The cellular pathology scheme is subdivided into general, veterinary and neuropathology.

Accreditation standards require action be taken by poor performers to improve the quality of their preparations. Most schemes offer expert assistance

Alcian blue 8G



Crystal violet



Dye	Ionic weight	Log P
Alcian blue 8G	1380	−9.7
Crystal violet	372	+1.9

Figure 9.1 Structural formulae of two widely used basic dyes, plus numerical descriptions of certain of their physicochemical properties.

iodine complexes. Non-ionic dyes must be mounted in aqueous media.

Why are the stains not taken up into every part of the tissue?

This question of selectivity is fundamental to histochemistry, and even routine oversight methods such as hematoxylin and eosin (H&E), Papanicolaou and Romanowsky-Giemsa stains distinguish nuclei from cytoplasm. So we must discover what factors control such selectivities.

Numbers and affinities of binding sites

Both these factors influence staining. However, in the absence of quantitative investigation, they are not readily distinguishable – so will here be discussed as a single effect. Stain-tissue affinities and numbers of binding sites present in tissues can vary independently.

Sudan dyes can be used as an example. These have a high affinity for fat but low affinity for the surrounding hydrated proteins. Alternatively one may consider staining systems in which covalent bonds are formed. Reagents give colored products only with a limited range of tissue chemical groupings. Thus, the acid hydrolysis-Schiff reagent sequence of the Feulgen nucleal technique gives red derivatives only with DNA.

An understanding of staining systems often requires consideration of patterns of affinities. With the traditional acid dye-basic dye pairs (H&E, Papanicolaou, and Romanowsky) the negatively charged acid dyes have high affinities for tissue structures carrying cationic charges (proteins, under acidic conditions). However they have low affinities for structures carrying negative charges (those rich in sulfated glycosaminoglycans, or in phosphated nucleic acids), with the opposite being the case for basic dyes. This produces two-tone staining patterns in which cytoplasm contrasts with nuclear material.

Practical staining conditions maximize selective affinities. Basic dyes are applied from neutral or acidic solutions, since under alkaline conditions proteins carry an overall negative charge and so may also bind basic dyes. Affinities are also influenced by varying the concentration of inorganic salt present. The various aluminum-hematoxylin, for instance, differ substantially in this regard. The critical electrolyte concentration methodology (Scott 1973) and several other empirical procedures are based on control of electrolyte content. However, staining that distinguishes two structures is still possible even when stain-tissue affinities and the number of stain-binding sites are the same. This is because rate of reagent uptake, or rate of subsequent reaction, or rate of loss of reagent or product, may not be the same in the two structures.

The hematoxylins and eosin 10

John D. Bancroft • Christopher Layton

Introduction

The hematoxylin and eosin stain (H&E) is the most widely used histological stain. Its popularity is based on its comparative simplicity and ability to demonstrate clearly an enormous number of different tissue structures. Hematoxylin can be prepared in numerous ways and has a widespread applicability to tissues from different sites. Essentially, the hematoxylin component stains the cell nuclei blue-black, showing good intranuclear detail, while the eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange, and red. While automated staining instruments and commercially prepared hematoxylin and eosin solutions are more commonly used in today's laboratories for routine staining, students of histological techniques should have a basic knowledge of the dyes and preparation techniques in order to troubleshoot and/or modify procedures for specialized use. It should be noted that hematoxylin has additional uses beyond just the hematoxylin and eosin combination.

Eosin

Eosin is the most suitable stain to combine with an alum hematoxylin to demonstrate the general histological architecture of a tissue. Its particular value is its ability, with proper differentiation, to distinguish between the cytoplasm of different types of cell, and between the different types of connective tissue fibers and matrices, by staining them differing shades of red and pink.

The eosins are xanthene dyes and the following types are easily obtainable commercially: eosin Y (eosin yellowish, eosin water-soluble) C.I. No. 45380 (C.I. Acid Red 87); ethyl eosin (eosin S, eosin alcohol-soluble) C.I. No. 45386 (C.I. Solvent Red 45); eosin B (eosin bluish, erythrosin B) C.I. No. 45400 (C.I. Acid Red 91).

Of these, eosin Y is much the most widely used, and despite its synonym it is also satisfactorily soluble in alcohol; it is sometimes sold as 'water and alcohol soluble'. As a cytoplasmic stain, it is usually used as a 0.5 or 1.0% solution in distilled water, with a crystal of thymol added to inhibit the growth of fungi. The addition of a little acetic acid (0.5 ml to 1000 ml stain) is said to sharpen the staining. Differentiation of the eosin staining occurs in the subsequent tap water wash, and a little further differentiation occurs during the dehydration through the alcohols. The intensity of eosin staining, and the degree of differentiation required, is largely a matter of individual taste. Suitable photomicrographs of H&E-stained tissues are easier to obtain when the eosin staining is intense and the differentiation slight (at least double the routine staining time is advisable). Ethyl eosin and eosin B are now rarely used, although occasional old methods specify their use: for example, the Harris stain for Negri bodies. Alternative red dyes have been suggested as substitutes for eosin, such as phloxine, Biebrich scarlet, etc.; however, although these substitutes often give a more intense red color to the tissues, they are rarely as amenable to subtle differentiation as eosin and are generally less valuable.

Under certain circumstances eosin staining is intense and difficulty may be experienced in obtaining adequate differentiation; this may occur after

Immunohistochemistry quality control

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Tracy Sanderson • Gregory Zardin

Introduction

Since its introduction into routine histopathology in the 1980s, immunohistochemistry has become established as an integral part of the diagnostic process. The use of immunohistochemical staining has developed within the diagnostic arena of an increasing range of infectious, neoplastic and reactive disease processes. Immunohistochemistry is now also able to provide prognostic or predictive information such as the likely response to specific treatments. Examples of such 'pharmaco-diagnostic' markers include estrogen (ER) and progesterone (PR) receptors and HER2/neu overexpression as well as CD117 (c-Kit) and CD20.

Reflecting the increasing diagnostic and prognostic role played by immunohistochemistry, the markers are known to generate results which may directly impact upon the patient care pathway. Consequently, it is vital that these investigative procedures are properly controlled by monitoring quality, both internally and externally.

External quality monitoring of clinical laboratories is achieved by participation in an external quality assurance scheme, such as UKNEQAS and NordiQC. Whilst in some countries participation in such schemes is a mandatory requirement in order to gain laboratory accreditation, the function of these schemes is primarily to monitor and improve the performance of the participating laboratory over a range of immunohistochemical tests.

It is important to ensure the correct internal quality control measures are in place. Immunohistochemisty is a complicated process and it is essential that the biomedical scientist has a sound understanding of all the requirements and procedures involved. There should be staff experienced in identifying and resolving associated diagnostic procedural problems in order to be able to provide effective and efficient quality control within the laboratory. In addition to technical understanding, the laboratory scientist should also have knowledge of the expected staining patterns for the antibodies in both pathological and non-pathological tissues. Good communication between the biomedical scientist and the pathologist must be maintained, particularly during the introduction and validation of new antibodies and procurement of positive control material.

Detailed documentation and an audit trail throughout the process are necessary for potential backtracking and troubleshooting. Such audit trail details can include antigen retrieval methods, antibody dilution data, control tissue samples, temperatures and incubation times. The advent of automated platforms for immunohistochemistry has improved this aspect of quality control, but vigilance is still required. These automated platforms generally use standardized protocols for antigen retrieval and staining procedures, which makes overall control of the process easier. The generation and storage of automated run logs by these platforms make full reagent traceability possible. The logs can also be interrogated in the event of abnormal staining to identify errors such as missed steps due to low reagent levels.

Factors affecting stain quality

Tissue factors

Fixation

Tissue fixation has a significant influence on immunohistochemistry as most antigens are altered during

this process (Williams et al. 1997). The purpose of fixation is to preserve tissue and prevent further degradation by the action of tissue enzymes or micro-organisms. As discussed in an earlier chapter, good fixation requires tissue to have adequate time in the fixative to allow the solution to penetrate, retaining uniform cellular detail throughout the tissue. However, in the routine laboratory this ideal may be compromised as it is difficult to define a standard tissue size, fixation time, and fixative for each specimen type. Tissues need to be adequately, but not over-fixed, so that antigenicity is preserved without excessive alteration. Prolonged fixation can result in the irretrievable loss of many antigens, particularly membrane-associated antigens such as CD20 and immunoglobulin (Ig) light chains (Miller et al. 1995; Ashton-Key et al. 1996). Lack of adequate fixation, or delay in fixation, may also be equally detrimental to labile antigens (Donhuijsen et al. 1990; von Wasielewski et al. 1998; GEFPICS-FNCLCC 1999).

Any fixative used must be compatible with immunohistochemical staining methods and formalin is still the most universal of fixatives. Formulations differ between laboratories and include 10% neutral buffered formalin (NBF), 10% formalin in tap water, 10% formal saline, and 10% NBF with saline (Angel et al. 1989; Williams 1993; Williams et al. 1997). Even though it may be the pathologist's choice, it can create a challenge for demonstrating certain antigens. Dabbs (2006) characterizes formalin as:

a satisfactory fixative for both morphology and immunohistochemistry provided that a simple and effective antigen retrieval technique is available to recover those antigens that are diminished or modified.

Williams et al. (1997) investigated the effect of fixation on immunostaining to establish whether a specific preparation schedule would allow for the optimal demonstration of all antigens. Of the fixatives tested, 10% formal saline, 10% NBF (except for CD45RO), and 10% zinc formalin (except for CD3) gave the most consistent results overall and showed excellent antigen preservation. More recently, alcohol-based fixatives have been considered as an alternative to formalin (van Essen et al. 2010) and produced satisfactory immunohistochemistry staining. Other fixatives which may still be used in some laboratories include Bouin's, B5 (mercury), zinc formalin, 10% formal-acetic and Carson's, which also have an influence on the reproducibility of staining, each presenting a change in pH, length of required exposure and different artifacts.

Fixatives dictate many factors for immunohistochemical staining, such as dilution, antibody incubation time, retrieval method (if applicable), type of retrieval solution, and special pretreatments (e.g. pigment removal). Depending upon the type of fixative used, the protocols may require slight modifications. With the advent of heat-induced epitope retrieval (HIER) (Shi et al. 1991) many of the problems associated with fixation have been reduced and, in conjunction with automated techniques, good-quality staining is achievable on most tissue sections.

Processing

As with fixation, all tissue must be appropriately processed to produce successful immunohistochemical staining. Tissue that is inadequately processed will potentially produce poor-quality sections, with poor adhesion to the slides – especially fatty tissue such as breast and skin. Modern tissue processors all have the option to include vacuum and temperature variation at each step, allowing for greater optimization of the procedure. However, high temperatures can be detrimental to antigens that are heat labile. It is recommended that paraffin with a low-temperature melting point be used for this reason.

Regarding paraffin processing of tissues for immunohistochemistry, as with fixation, there is no standard protocol for the optimal demonstration of all antigens, as concluded by Williams et al. (1997). In a study of laboratories in the UK, Williams (1993) found nearly as many different schedules as the number of laboratories participating in the survey. Of the nine tissue-processing factors investigated, only two had any significant effect on immunoreactivity. Increasing the temperature of processing from ambient to 45°C, as well as longer processing times for dehydration and wax infiltration, were both found to improve immunostaining. Other

factors including type of processor, type and quality of reagents, time in clearing agent, use of vacuum, most of which had been suggested as possible causes of poor processing (Horikawa et al. 1976; Trevisan et al. 1982; Anderson 1988; Slater 1988), were found to have no effect on subsequent immunohistochemistry.

Microwave processing is now being introduced into some laboratories to speed the processing time and reduce turnaround time for diagnostic specimens, and has been used successfully in conjunction with routine antibody staining. Acceptable staining was achieved when compared to tissues processed in a conventional processor (Emerson et al. 2006). As with all processing, if the tissue is not completely fixed then artifacts will be introduced.

One important point is that any control tissues used in the laboratory should be processed using the same protocols established for patient samples.

Reversal of fixation/epitope retrieval

The quality and reproducibility of immunohistochemical staining relies upon the reversal of fixation, which results in the targeted epitope being exposed, allowing for the antigen binding site to be available. The revolution of reversing the hydrogen crossbonds formed by formalin was introduced by Shi et al. (1991). There are now numerous methods for epitope retrieval including protein digestion techniques or, more commonly, heating the slides in a buffered solution. Cattoretti et al. (1993) introduced the solution most commonly used in standardized retrieval methods. They used a citrate buffer at pH 6.0, which is inexpensive, stores easily, and is readily available commercially or easily prepared in the laboratory. Other buffers used include EDTA-based solutions at a higher pH range, which produce more intense staining of some antibodies. These methods have allowed for the successful demonstration of a much greater range of antigens in tumors, including proliferation markers and oncogene expression. The use of automated immunostainers has brought greater standardization of retrieval methods, as these use standard retrieval solutions with defined reproducible protocols. Non-automated laboratories may have a number of variables that require internal standardization in the antigen retrieval technique including the choice of heating method (e.g. pressure cooker, microwave, etc.), retrieval solution, pH, temperature, volume of the fluid, and the temperature and exposure time while heating and cooling slides.

Equipment commonly used to perform epitope retrieval includes the modified pressure cooker, initially reported by Norton et al. (1994), microwaves, waterbath or a pretreatment module. Some automated platforms have on-board retrieval where individual slide bays can be heated with the appropriate solution on the slide.

Other factors required for successful retrieval include the proper drying and complete removal of water from slides. In addition to avoiding wrinkles or tears in the tissue, these factors will all assist the adhesion of tissue to the slide. With respect to enzymatic proteolytic ‘epitope retrieval’, such as trypsin digestion prior to immunostaining, the choice of enzyme usually dictates the temperature and pH of the solution, as different enzymes have different preferential pH and temperatures. For example, the optimal values for a mammalian-derived trypsin are pH 7.8 at 37°C, with 0.1% calcium chloride included as an activator (Huang et al. 1976). The concentration of enzyme required is dependent on the proteolytic qualities of the product being used. A typical concentration used for many commercial trypsins employed in immunohistochemistry protocols is 0.1%. The concentration, pH, and temperature are then usually held constant, while the time of digestion is varied. The time required for optimal digestion will vary, depending on the antigen under investigation, the quality (proteolytic capabilities) of the trypsin and the length of formalin fixation. For antigens that are only present in small amounts, e.g. immunoglobulin light chains on the surface of B cells, the time for optimal digestion may vary from case to case, depending on how long each case has been fixed in formalin.

Reagent factors

Production of high-quality staining is dependent upon the correct storage, handling and application

of the reagents used. Once a protocol has been developed, it is important to ensure the reproducibility of the stain. To achieve this, the storage conditions and expiration dates of in-house and commercial reagents must be monitored as the preparation and use of each reagent must be consistent. Details of the storage and preparation of all reagents used in each staining run must be documented as part of the audit trail to allow back-tracking and troubleshooting.

Reagent monitoring is one area in which the use of an automated staining system with bar-code reagent labeling can be of assistance both in alerting the operator to reagents which have reached their expiry date and in the automated creation of audit trails and quality control documentation.

Buffers and diluents

The buffer and diluent used for wash steps and antibody dilution will affect the results of immunostaining. The pH of these reagents needs to be monitored and must be checked and documented prior to use. If it falls outside of the range proscribed by the established protocol, corrections must be made or the reagent discarded.

Many antibody diluents contain additives such as sodium azide to stabilize and maintain the protein. Although this extends the shelf life of the antibody, the additives may interfere with, or inhibit, staining if present at excessive levels.

Antibodies

The storage temperature of an antibody is critical to its stability. Commercially available antibodies should be accompanied by a specification sheet, containing storage and handling instructions.

Concentrated antibodies tend to have a longer shelf life than pre-diluted 'ready to use' antibody preparations. Concentrated antibodies can be mixed with glycerine to prevent ice crystal formation, aliquoted into cryovials, then 'snap' frozen and stored in a −80°C freezer. This greatly extends their shelf life; compliance with the expiry date printed on the antibody packaging is important. The storage temperature for antibodies and reagents should also be monitored closely, as any fluctuation in temperature may cause increased deterioration of reagents. Frost-free −20°C freezers should hence be avoided for the storage of antibodies due to damage caused by the freeze-thaw cycles that these types of freezer perform.

Procedural factors

The automation of immunostaining is perhaps the easiest way of improving the reproducibility and consistency of the staining. Although the use of automation is increasing, many laboratories still perform at least some immunohistochemical staining manually. Other laboratories perform semi-automated staining (for example manual epitope retrieval). The production of good manual procedures is important for these stains and also for use in case of failure of automated staining machinery. These procedures must be clear, easy to follow and sufficiently detailed to ensure a minimum of inter-operative variation. Adherence to these protocols and the reduction of human error is the goal in order to ensure consistent stain quality.

Block and slide storage conditions

Processed blocks should be kept in a cool dry place. Resealing paraffin blocks following cutting can help protect the tissue from everyday elements such as air drying, excessive moisture or physical damage. The use of fresh cut controls for each run would be ideal but this is not always feasible in a busy laboratory. Repeated sectioning of a control tissue block can also result in loss of usable tissue, although some laboratories now have a control on the same slide as the test section.

Pre-cutting control slides is more time efficient and serial sections result in minimal tissue loss. The correct storage of pre-cut slides is important and frequently overlooked as a potential source of error in staining. Some studies have found deterioration of antigens in stored sections (Raymond & Leong 1990; Bromley et al. 1994; Prioleau & Schnitt 1995). Others have found no deterioration of some markers investigated, including estrogen receptor (ER), CD3, CD20, CD45RO, vimentin, and Ig light chains, in

sections stored for up to four months at room temperature (Williams et al. 1997; Eisen & Goldstein 1999).

The viability of the antigen and speed of antigen deterioration in cut tissue sections is highly dependent upon the antigen under consideration and the temperature used for section adhesion. For example, whilst antigens such as CD30 and PSA seem relatively robust, CD117 (C-kit) deteriorates quickly and should be cut fresh.

The temperature at which the slides are dried can also affect the immunoreactivity of the antigens. It is advisable to dry all slides for immunohistochemical staining at 37°C. Urgent cases can generally be dried at 60°C for up to 4 hours. The exception to this is HER2, where it is recommended that sections should not be dried at 60°C for more than 1 hour.

An appropriate number of control slides and blocks should be kept with these factors in mind. This stock level will vary depending on the workload in each individual laboratory.

Monitoring stain quality

Whenever a new batch of reagent is used, its details and the efficacy must be recorded, checked and compared with the previous batch. Reagents from different batches should never be mixed. Any enzymes used must be validated prior to use, due to a high degree of inter-lot variation. It should be noted that poor storage or shipping conditions can result in a reduction in enzyme activity. Antibody validation documentation should be available and is best kept in combination with the antibody specification sheet. This may be in either paper or electronic form. Detection system reagent validation is best performed by using a panel of several different antibodies. This should include different antigen retrieval methods and cover a variety of different staining patterns (nuclear, cytoplasmic, and membrane staining). A clean (i.e. non-reacting) negative control is just as important as the intensity of positive staining patterns, and such controls must be carefully checked.

Validation of antibodies

It is important that all antibodies used in diagnostic testing are fully validated in the laboratory and detailed records are kept of the validation process. This should include demonstration of the reactivity of the antibody, validation of the procedure and quality checking of positive and negative controls used with that antibody. Before introducing a new antibody into the laboratory repertoire it is important to research details of the clone required. Most commercial antibodies have data sheets available on-line which should include a number of facts for consideration. These should indicate the host in which the antibody was raised (e.g. rabbit, mouse, or goat), location of the target antigen, concentration of the antibody, recommended application (e.g. frozen tissue, formalin-fixed paraffin-embedded tissue), recommended positive and negative control tissue sources, classification (e.g. analyte-specific reagent, research use only, or *in vitro* diagnostic) and reference materials for the application of the antibody. The specification sheet also typically includes suggested staining protocols.

Most antibodies used in diagnostic pathology laboratories are classified as 'research use only' or 'analyte-specific reagent'. This means that it is the responsibility of the diagnostic laboratory to validate and document the sensitivity and specificity of the antibody. This process can be simplified if an appropriate antibody classified for '*in vitro* diagnostic' (IVD) use can be sourced. The vendor has assumed responsibility for the validation and application of these antibodies.

The suggested protocol should serve as a baseline whilst working up the antibody and each laboratory should optimize the stain. If no protocol is suggested by the manufacturer, journal articles can be a good source of baseline protocols.

Selection of the most appropriate epitope retrieval method is important to ensure the maximum sensitivity of the stain. Antibodies are pH sensitive and it has been shown that staining intensity is better when the epitope retrieval step is performed at a pH specific to each antibody. It has also been shown that an antibody may not be specific or work as well if a

proper pH is not maintained during the retrieval step. Selection of the detection complex used may also be influenced by the primary antibody. Some antibodies have been found to work better with an alkaline phosphatase detection system than with a horseradish peroxidase system and vice versa. Antibodies commonly used in pigmented tissues such as melanoma markers may benefit from a red (or other) chromogen end-point, as the endogenous pigment can disguise the more commonly used brown DAB chromogen.

Staining protocol development generally consists of trial and error, making sequential alterations in order to achieve optimal signal-to-noise ratio. In real terms this means strong, crisp target antigen staining with little or no background staining. If the staining is too strong, then further dilution of the primary antibody may improve staining specificity. If target antigen staining fades and the background remains, then the addition of a blocking step, change of epitope retrieval or changing the detection system used (e.g. to a polymer-based system) may improve the result. Changing the antibody diluent may help to increase the antibody's reactivity, while at the same time lowering background staining. Weak staining may be improved with an increase of the antibody concentration (e.g. from 1:50 to 1:25).

Negative staining can be more difficult to resolve and may be the result of multiple factors. It is advisable to rule out human or mechanical error by repeating the staining before making further modifications. If the stain remains negative, change one variable at a time and document each reagent, step and reaction time. Negative staining may be resolved by increasing the antibody concentration, changing the epitope retrieval method, changing the solution pH, changing the antibody diluent to one of a different pH, changing the base composition or using an amplification step as part of the detection system. It is also important to ensure that the tissue stained should exhibit positive staining and the slides are freshly cut.

The duration of formalin fixation to which the tissue has been exposed may affect the reversal of protein cross-linking. Over-fixation of tissue may require more aggressive epitope retrieval methods to achieve satisfactory retrieval. This must be considered if the control or test tissue available for protocol development and validation has been stored in formalin for an extended period of time.

The final stain protocol must be confirmed on both positive and negative tissue controls prior to implementation. False-positive staining of negative control tissue suggests that the concentration of the antibody is too high.

Once an antibody dilution and staining method has been established and validated, each step of the procedure must be clearly documented and maintained in written or electronic form. This should include the antibody lot, expiration date, dilution, details of blocking steps performed (serum, avidin-biotin, and hydrogen peroxide), secondary and label (detection), chromogen tested, and the duration of each step.

Control slides

Control slides can include both reagent substitution and internal or external tissue controls. A positive control for each antibody should be used with every staining run. It is important to obtain a reliable, known, positive control tissue for use with each antibody offered by the laboratory. Control tissue selection should be supported by publications and the selection process should be documented. Many commercial antibody specification sheets make recommendations regarding controls, and most cite references which are of use when selecting control tissue.

The acquisition of appropriate control tissue requires knowledge of the desired tissue type (e.g. kidney, liver, etc.) and whether the target antigen is expressed in normal or tumor tissue. It is helpful if the person validating a control has knowledge of the prospective control tissue's diagnosis in the case of tumor controls to assist in the evaluation of expected staining patterns. In order to be comparable to the test section it is important that the fixation and processing of control tissue should be the same as the patient tissue being tested. Ideally, target antigens in positive controls should be distributed across the

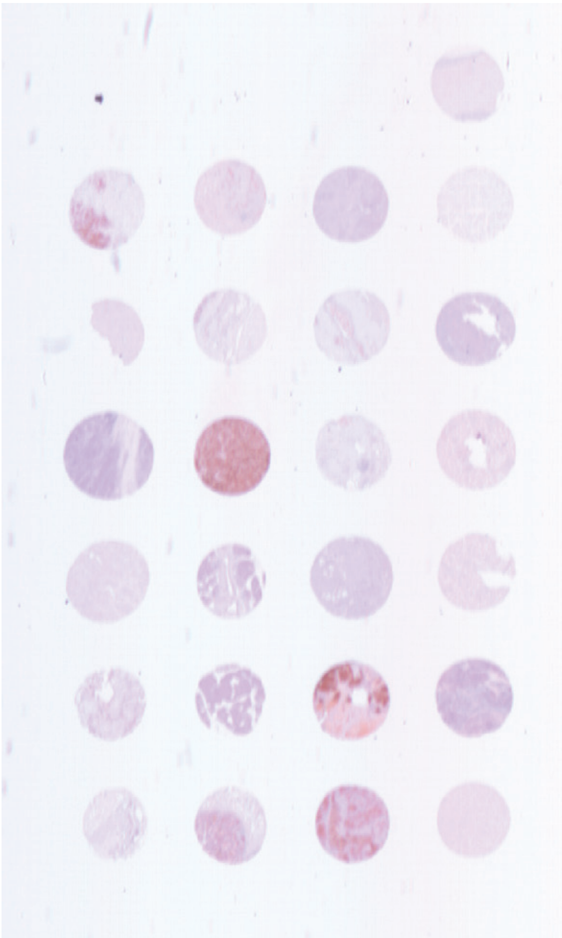

Figure 20.1 Low-power image of a multi-tissue control stained with an antibody to polyclonal carcinoembryonic antigen (CEA). Using carefully chosen 'donor' tissues, both positive and negative tissue controls can be demonstrated on the same slide.

entire sample and, if possible, should be present at a range of densities in order to monitor the sensitivity as well as specificity of the stain.

Whilst a separate positive control should be included for each antibody, a single tissue type is often suitable for use as an external control for several different antibodies. For example, a section of appendix can be used for testing antibodies to low molecular weight cytokeratins, EMA, vimentin, desmin, SMA, CEA, S-100, NSE, CD45, CD20, CD3, CD4, CD8, CD79a, bcl-2, Ki-67, etc. (Balaton 1999). One way of creating a control block containing

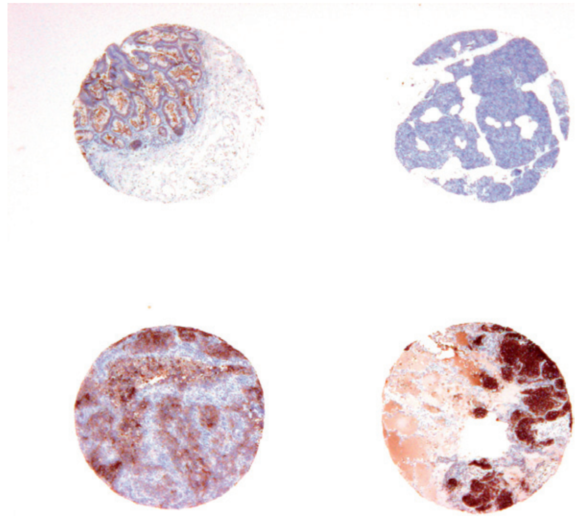

Figure 20.2 High-power image of a multi-tissue control stained with an antibody to polyclonal CEA, demonstrating both normal tissue and tumor staining, in addition to a negative normal tissue core.

multiple positive and negative tissues suitable for a wide range of antibodies is to use a tissue microarray (Figs 20.1 and 20.2). A composite block, containing representative punches from multiple tissue types in a single block, can be used for the majority of stains offered by a laboratory. With careful tissue selection the block should not have to contain an overlarge number of punches in order to achieve this goal. A drawback of this approach is that, whilst it simplifies daily quality control, it requires a large amount of available control material, particularly in larger laboratories offering a wide range of antibodies.

Internal and external positive controls

Many tissues contain native components that serve as internal positive controls for immunohistochemical staining: e.g. the crypts of normal colon stain with polyclonal CEA (Fig. 20.3). These internal controls are in many ways better than external tissue controls, since they demonstrate that the test tissue was appropriately fixed and processed. Although external positive tissue controls do not show this, they are required for some tests where there is no

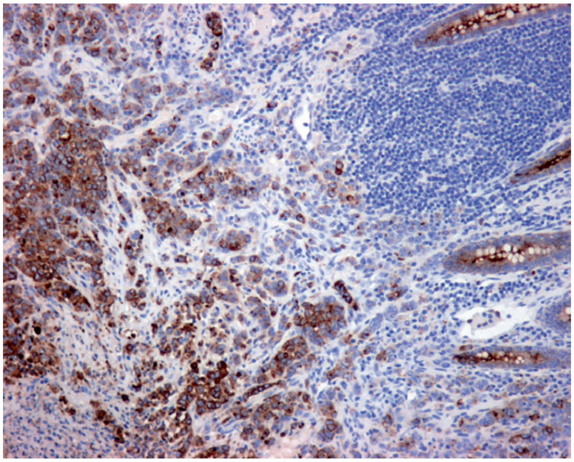

Figure 20.3 Section of colon, demonstrating staining of normal crypts for polyclonal CEA, serving as an internal positive control.

internal positive control in the tissue under investigation, such as those performed for infectious agents. External controls are also needed whilst working up an antibody for inclusion into the laboratory repertoire, and are also of use in assisting the day-to-day monitoring of the stain where it is preferable to use the same tissue with a known pattern of staining. If an internal control is not present, and/or if there is doubt over the retention of antigenicity in the test tissue, then this can be checked by staining with vimentin. However, since vimentin is a relatively robust antigen it is possible that its viability could be maintained whilst more labile antibodies such as CD3 may be lost.

Daily slide review

All slides should be reviewed and quality assessed prior to being sent out of the laboratory. This can be performed by an appropriately trained laboratory scientist or a pathologist. The reviewer should be able to distinguish acceptable signal-to-noise ratio and recognize interpretable results. At a minimum, the positive control slides should be screened for expected results.

Without stained controls the results of many immunohistochemical assays cannot be validated. The controls should also demonstrate the sensitivity (i.e., tissue with low expression of the target antigen stains positive) and specificity (i.e., negative controls are not stained and background staining is absent) of the stain. Controls indicate whether the staining protocols have been followed correctly, whether day-to-day and worker-to-worker variations have impacted upon stain quality, and whether the reagents used continue to be in good working order.

External quality assurance

Whilst daily internal monitoring of stain quality through the use of control slides is vital, it is also important to ensure inter-laboratory consistency of quality and results. This is best achieved through participation in an external quality assurance scheme such as UKNEQAS or NordiQC. These schemes are arranged into different modules, each covering common stains performed in different areas of pathology. Examples of modules are breast pathology (HER2, ER, PR, etc.), neuropathology (NFP, GH, TSH, etc.) and general pathology (desmin, CD3, Ki67, etc.). Participants choose which modules they wish to participate in, and are sent slides to stain with relevant selected antibodies. The stained slides are returned to the scheme organizer often accompanied by one of the laboratories in-house control slides used for the requested antibody. Submitted slides are assessed and scored independently by the scheme organizer and participating laboratory, who will then receive both individual feedback on the slides submitted and general feedback on results from all participants. The methods used to produce the highest scoring slides are taken as 'best practice' and made available for all laboratories to consider. Any laboratory producing consistently poor results will be offered advice by the scheme organizers to help improve future results.

Through participation in such schemes the laboratory can assure service users that the results they produce are not only consistent but also that they are in concordance with the national consensus. This is particularly important for laboratories using prognostic and predictive markers which may impact on patient care (Rhodes et al. 2001; Ibrahim et al. 2008; Bartlett et al. 2009). In many countries participation

in such schemes is a prerequisite for the accreditation of the laboratory.

Troubleshooting

Troubleshooting problems in immunohistochemistry can be difficult due to the complexity of the technique and the number of variables involved. However, good documentation of the processes involved can help with backtracking to potential sources of error. The use of bar codes on slides and reagents on automated staining systems has helped to reduce some of the common human errors, but machines are not entirely reliable and have their own associated problems. One of the advantages of an automated system is the ability to see on screen records of staining logs and reagent preparation details, which can help to identify problems promptly.

A key element to producing good-quality immunohistochemical staining is in the preparation of the slides. Poorly fixed or processed tissue tends to be more difficult to section, being prone to detachment from the slide, especially given the harsh nature of some pretreatment protocols. Furthermore, poor section quality can lead to problems with interpretation if visualization of the staining is occluded or the tissue morphology is damaged. This includes section thickness, damage (such as holes or scores) and excessive heat exposure (causing dry sections). An experienced laboratory scientist with a good understanding of all the processes and techniques involved should be able to identify and resolve all of the problems that may arise.

The most common problems that are likely to occur are either false-negative or false-positive staining, and the potential sources of error for these are discussed below.

False-negative staining

The absence of staining of an antigen that should be present in the tissue can present in various patterns. The easiest to identify is when the positive control slide and the patient (test) slide are completely negative. After checking that the correct positive control section has been used, the source of the problem needs to be identified and rectified, before the stain is repeated.

In the second type of false-negative staining the positive control slide is negative, but the patient slide shows positive staining in either the area of interest, or in some internal component that acts as a positive control. If this internal control stains according to the expected pattern it may not be necessary to repeat the stain as long as the failure of the control is documented. The cause of the failure of staining in the positive control still needs to be determined to ensure it is still viable to be used in future staining runs.

The third pattern of false-negative staining occurs when the positive control slide stains appropriately, but the patient slide is completely negative. This is more difficult to detect because at first glance it may appear that the patient test is just negative. In this instance it is important to check for any internal positive control components within the tissue, and if these are also negative the stain should be repeated. If the negatively stained section was part of a panel of antibody tests, then it may help with the assessment if the negative result is appropriate when compared to the other test results. A number of factors can cause false-negative staining.

Process failure

This can be caused by human or mechanical error if there is automation of the process. Human errors usually occur because the standard procedure has not been followed, but commonly such errors involve missing a step, or performing steps out of sequence, incorrect reagent preparation, or reagent incubation time not being delivered. Shortening steps may not allow for chemical or immunological reactions to complete.

Process automation has cut out some potential errors by bar-coding slides and reagent bottles. However, it is still possible for slides to be incorrectly labeled, or for reagents to be wrongly prepared unless they are commercial, ready-to-use reagents. Another potential problem is reagents

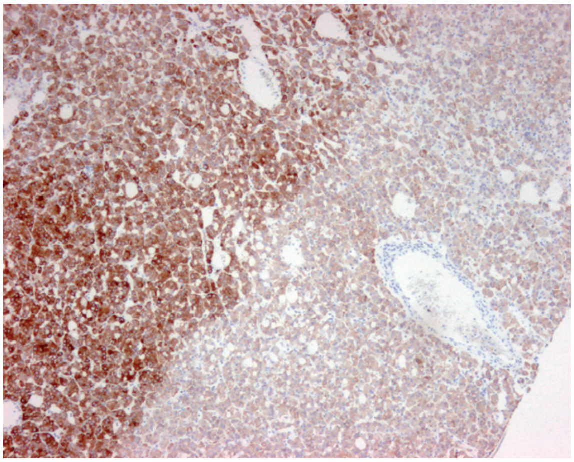

Figure 20.4 Adenocarcinoma stained on a horizontal immunostainer with an antibody to cytokeratin 7, demonstrating incomplete coverage of the tissue section by staining reagent.

being skipped if the instrument runs out of wash buffer, thereby compromising staining. Incorrect programming of an instrument can occur, but should be picked up before a staining run is started. A common artifact that can be seen is uneven staining of the section caused by incomplete coverage by one or more of the reagents (Fig. 20.4).

Instrumentation failure can also occur through pump failure, clogged or damaged probes, or because electrical components wear out. The computer can have software glitches or experience external network problems if interfaced with a laboratory or hospital system. Equipment should be well maintained and regular maintenance is helpful to keep instruments in good working order.

Positive control selection

Positive control material should have previously been tested for validation before use. If the positive control slide is negative and the patient slide positive, there are three main problems that could have occurred. Firstly, the wrong control could have been used, which may happen when inexperienced staff members are working in the laboratory. It can help to clearly label pre-cut control sections with the antibody or antibody group they can be used for, such as cytokeratins, or to have a list of antibodies and the appropriate control section required. The second problem might be that the area of interest in the control slide might have been cut through. This can be prevented by testing the first and last slide of each batch of sections, in order to validate that the control is still demonstrating appropriate staining. Thirdly, some antigens are more labile, and their antigenicity deteriorates with time in cut sections, resulting in weak or negative staining. If this is suspected, a fresh section should be cut and stained to make sure the block still contains viable control tissue. Once any of these antigens have been identified or there are antibodies that are used infrequently, it is advisable for the laboratory to only keep a small stock of pre-cut sections, or to cut them freshly as required in order to prevent further occurrence.

Incomplete deparaffinization

Failure to remove paraffin wax from the slide may interfere with the ability of the antibody to penetrate the tissue and thus may inhibit antibody binding. Reagents used for de-waxing and dehydration should be changed regularly, and paraffin wax with a high plastic content may require longer times in xylene with agitation to assist with complete removal. Some pretreatment solutions de-wax and pretreat in one step. It is important to monitor the use and temperature of the reagents and follow the manufacturer's recommendations for use to ensure that they continue to perform at an optimal level. Changing or rotating solutions regularly is recommended to ensure that deterioration of these reagents does not introduce unnecessarily weak or false-negative staining, which is often overlooked as a potential problem.

Epitope retrieval

It is essential that the correct epitope retrieval protocol has been performed on the sections, as not all retrieval solutions work for all antibodies used in the laboratory. For example, the antibody BerEP4 will generally not work if pretreated in high pH EDTA-based retrieval solutions. The retrieval protocol should have already been defined as part of the optimization of a new antibody and should be

strictly adhered to; otherwise, it will most likely result in false-negative staining. Therefore, slides should be clearly labeled and sorted for the specific retrieval procedure required. This is generally less of an issue on automated platforms as the retrieval step is part of the set staining protocol. The retrieval will either be carried out on the machine, or a slide label will have been attached to the slide with the appropriate protocol details printed on it, making it easier to indentify the correct pretreatment.

When HIER protocols are performed, it is advisable to monitor the temperature of the solution to check that it has reached the correct setting, and that it has been maintained for the required length of time. Failure so to do may result in substandard reversal of fixation and possible negative staining. Many of the automated systems are able to monitor and log retrieval temperatures electronically and can be easily viewed on screen.

If enzyme digestion is employed as the retrieval method, care should be taken to ensure that the correct time and temperature protocol is used depending on the digestion agent and the antibody in question. Over-digestion of the tissue can result in loss of morphology of the tissue, at which point the antigen may have been destroyed, resulting in negative staining.

Temperature

Chemical reaction rates are affected by temperature, so it is important to monitor all aspects of the procedure where heating or cooling is required, either during pretreatment or staining. Some automated stainers use heat during various steps to speed up chemical reactions and these can develop faults. However, regular maintenance and monitoring should help to prevent these occurrences. Antibodies are proteins, and as such their structure can be modified by heat, which may decrease the sensitivity of antibody binding during the staining process. It is also important not to overlook the general room temperature of the laboratory where immunohistochemical staining takes place. With numerous fridges and staining machines working, the core temperature of the room can rise by several degrees if there is no air-conditioning system in place. Some staining machine manufacturers have recommendations about minimum and maximum advised working temperatures.

Antibody preparation

All commercially available antibodies should be labeled with an expiry date and come with a datasheet detailing the correct storage requirements of the antibody. Most concentrated and pre-diluted antibodies are recommended to be stored at 2–8°C. Those that are less stable may need to be aliquoted and frozen at –20°C. Antibodies stored at 2–8°C will still decline over a period of time, as oxidation occurs on exposure to air and at some point will decline rapidly and produce a false-negative result. This deterioration may be picked up by close monitoring of the positive control section if the expected level of staining starts to decrease.

Human error in the preparation of an antibody dilution may occur. Pipettes should be regularly maintained and calibrated at least annually. Staff should be trained in the correct mechanical use of the pipette, as well as choosing the appropriate size of pipette for a particular volume range. Antibody dilutions should be documented to include the date that the antibody dilution is made in order to identify when a possible preparation error may have occurred.

Chromogen incompatibility

Various detection systems are available for visualization of the antibody, and it is important to understand the compatibility of chromogen and enzyme label. The standard combination is horseradish peroxidase (HRP) enzyme used with diaminobenzidine (DAB) chromogen, but other chromogens such as amino ethyl carbazole (AEC) work with HRP as well. Fast red chromogen and BCIP/NBT react only with an alkaline phosphatase enzyme. Reading the manufacturer's recommendations for preparation and shelf life of a prepared chromogen is important. These problems should not occur on automated platforms, as the detection reagents are generally supplied as a ready-to-use kit with

bar-coded bottles that cannot be confused. When using alkaline phosphatase-based detection, care should also be taken from counterstaining to mounting as some enzyme labels are alcohol soluble and cannot be dehydrated.

False-positive staining

False-positive staining is often easier to troubleshoot than false-negative staining, although it is potentially much more serious. If the false-positive staining is interpreted by the pathologist as real (i.e. positive) staining, then the patient may be incorrectly or unnecessarily treated. An experienced laboratory scientist should be able to identify possible false-positive staining, and should then either repeat the test or bring it to the attention of the pathologist to discuss any action required. When false-positive staining is observed in a patient slide only, it may be due to different fixation or processing of the patient tissue. In these instances it may be helpful to run a negative patient slide alongside the repeat test to check for non-specific false-positive staining. As with false-negative staining, there are a number of factors which can cause the problem.

Poor quality of fixation

With greater emphasis on meeting targeted diagnostic turnaround times, the laboratory can be put under pressure to process samples through as quickly as possible. However, this can greatly impact the quality of tissue processing. When fixation and processing times are too short, tissue may not be adequately dehydrated. The result is that the tissue may be partially unprocessed, or that the center of the tissue will be alcohol fixed during processing. In this case the immunohistochemical staining pattern of the patient test tissue can be quite variable, compared to that of the control section on which the dilution and method have been developed (Fig. 20.5).

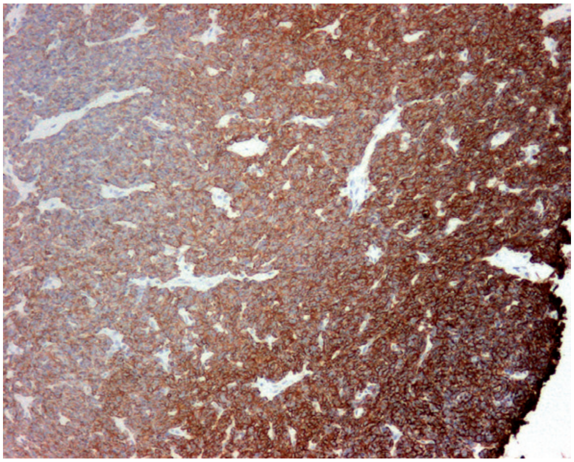

Figure 20.5 Section of poorly fixed thymus stained with an antibody to AE1/AE3 cytokeratin cocktail, demonstrating a gradient of staining from the formalin-fixed outer edge to the alcohol-fixed center.

Technical preparation

As mentioned previously, it is important to start with good-quality sections for immunohistochemical staining, as this is a common cause of false-positive staining. Poorly fixed and/or processed tissue is more difficult to cut and has a greater tendency to detach from the slide. Therefore, all sections should be picked up onto positively charged slides to assist with tissue adhesion and to prevent detachment during potentially harsh pretreatments. They should all be cut at the same thickness and need to be as flat and wrinkle free as possible. The waterbath used for floating out the sections should be wiped after every case to prevent floaters and squamous cells being transferred to the next section (see Fig. 20.6).

Poor section quality can result in streaks, overall blushing across the tissue, or patches of non-specific positive staining. Calcified tissue can cause scores or holes which may affect staining, but any decalcification treatment must be kept to a minimum as the acidic reagents can be deleterious to the end result. Wrinkles, tears, and folds create areas in which the reagents are not properly rinsed away and remain trapped underneath or on top of the tissue (Figs 20.7–20.9), so that by the time the chromagen is applied those areas are intensified and may make it impossible to interpret the staining.

Once cut, the sections need to be dried on properly before staining to help with adhesion. Oven-drying must be monitored and maintained at a constant temperature, as tissue exposed to high temperatures

for long periods of time may demonstrate edge artifact (Fig. 20.10). If sections are not going to be stained immediately, they should be dried at 37°C and then stored at room temperature once dried on.

Epitope retrieval

As discussed earlier, the use of retrieval solutions must be monitored to ensure the correct protocol is performed each time. Variations of pH or temperature of the solution will interfere with staining results. One possible effect of incorrect retrieval is non-specific staining due to the amplification of endogenous biotin in the tissue (O'Leary 2001), which is observed with avidin-biotin detection systems. Some antibodies do not require any pretreatment, and exposure to a retrieval solution or step may create non-specific nuclear staining (Fig. 20.11).

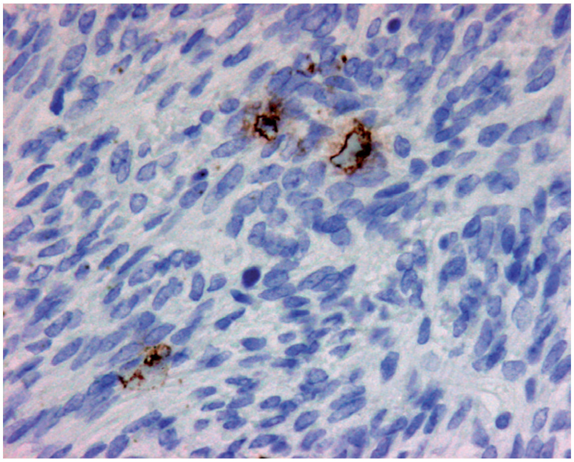

Figure 20.6 Section of lymph node stained with an AE1/AE3 cytokeratin antibody cocktail, demonstrating squamous cell contaminants that result from the technician placing ungloved fingers into the water bath, also known as floaters.

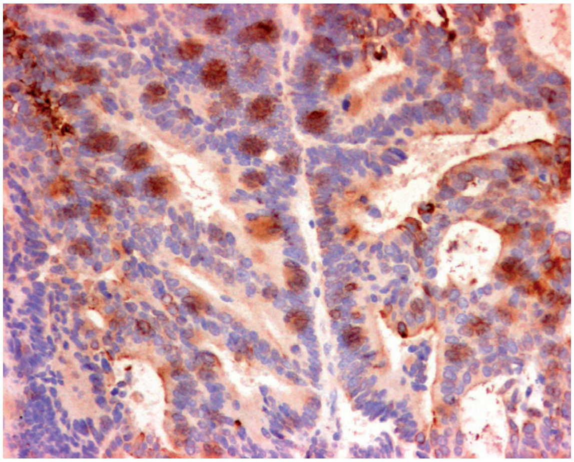

Figure 20.8 Section of colon, demonstrating trapped chromogen due to poor tissue adhesion to the slide.

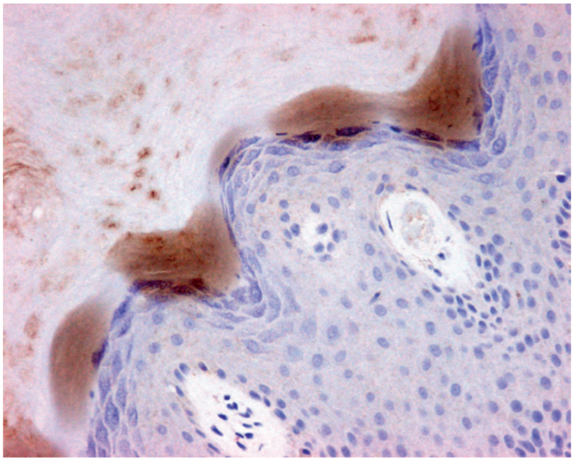

Figure 20.7 Section of skin intended as a negative control, demonstrating trapped chromogen under the keratin layer due to tissue lifting.

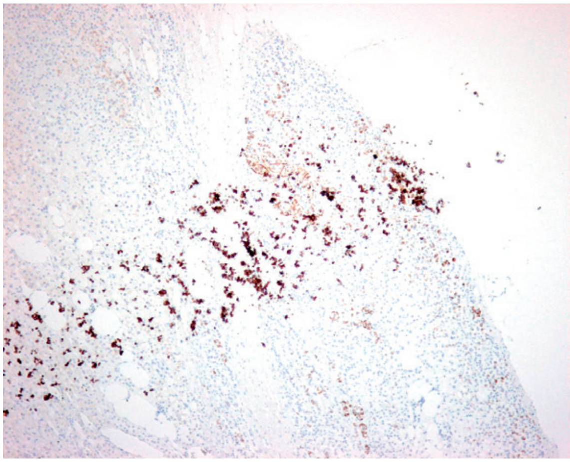

Figure 20.9 Section of skin stained with an AE1/AE3 cytokeratin antibody cocktail, demonstrating chromogen streaking across the section due to poor rinsing. Staining is present across an area expected to be negative.

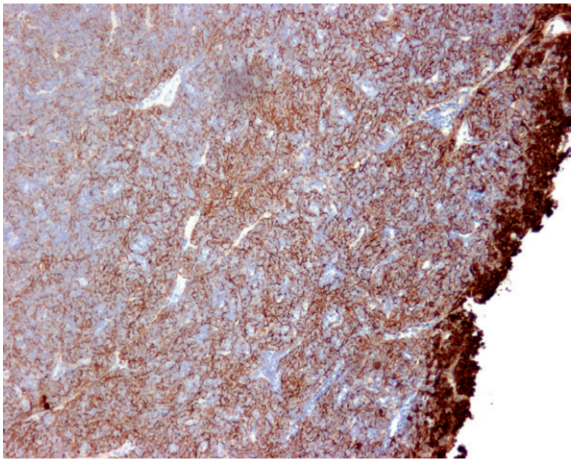

Figure 20.10 Tissue section, demonstrating edge artifact due to excessive drying.

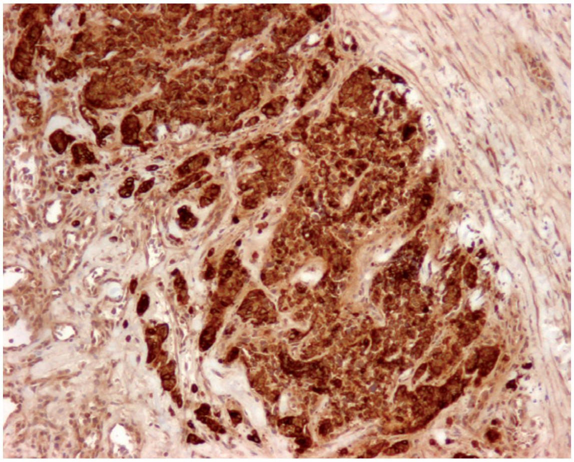

Figure 20.12 Over-digested carcinoma stained with an AE1/AE3 cytokeratin antibody cocktail. Excessive staining creates difficulty in identifying true positive staining.

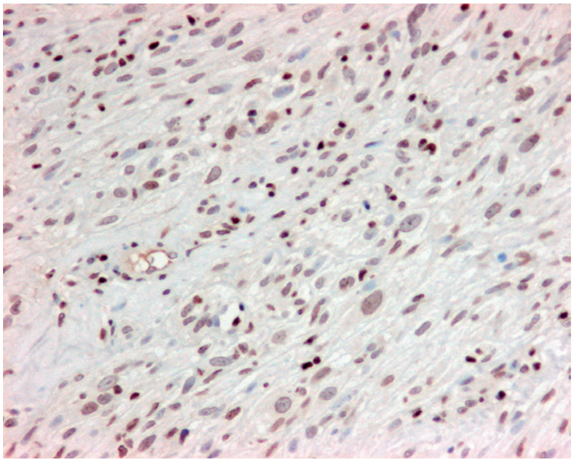

Figure 20.11 False-positive nuclear staining demonstrated with an antibody to polyclonal myosin due to unnecessary heat-induced epitope retrieval.

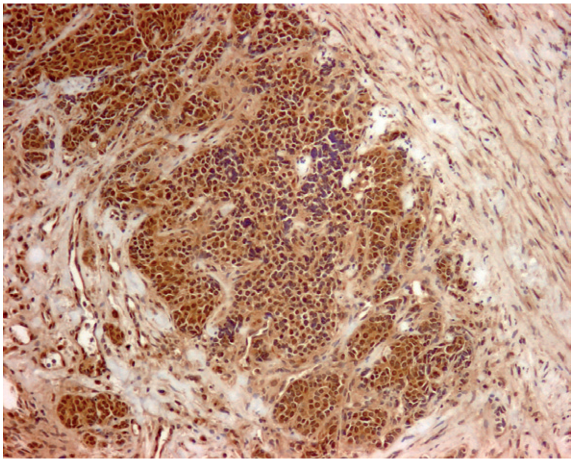

Figure 20.13 Over-digested carcinoma (same case as in Fig. 20.12) intended as a negative reagent control slide, confirming that much of the staining observed in the patient AE1/AE3 section is non-specific staining.

Care should be taken not to over-digest the tissue when using proteolytic enzymes. This can be caused by extending the time beyond that determined to be the optimal digestion time within the individual laboratory, or by performing the digestion step at a warmer temperature than has been determined in the laboratory's validation process. Excessive heat will typically increase the rate of digestion. If the section is not sufficiently rinsed following the digestion step and the enzyme is not removed completely, it will continue to digest the tissue. All of these factors lead to over-digestion of proteins, which may then diffuse into or deposit onto the tissue, leading to diffuse non-specific staining. The occurrence of over-digestion should be apparent by the subsequent damage to tissue morphology (Figs 20.12 and 20.13).

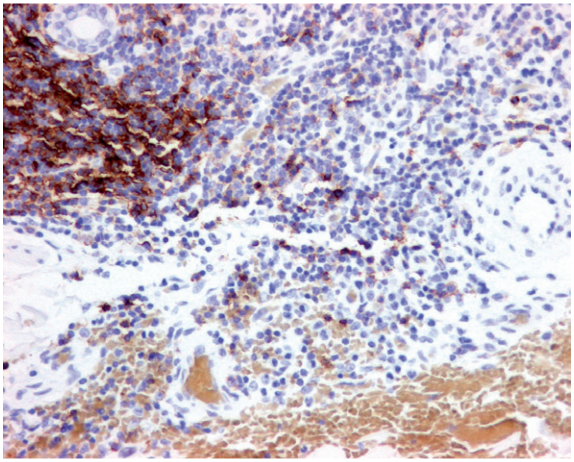

Figure 20.14 Lymphoid tissue stained with an antibody to CD3, demonstrating non-specific staining of red blood cells (intrinsic peroxidase activity) due to inadequate quenching with hydrogen peroxide solution.

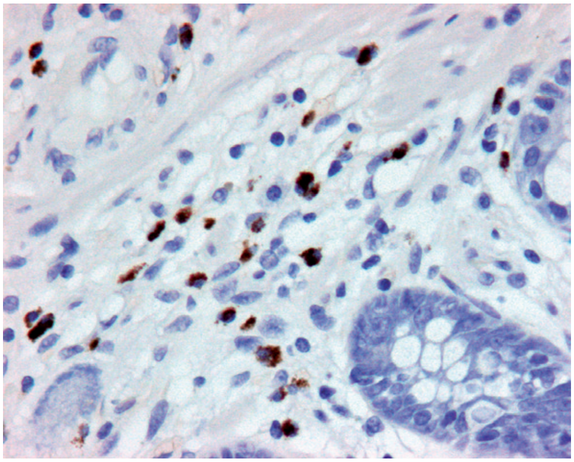

Figure 20.15 Section of colon intended as negative reagent control, demonstrating mast cell staining due to inadequate quenching of endogenous peroxidase activity within mast cells.

Tissue drying (wetting agents)

Once the tissue has been de-waxed for staining the sections should be kept moist and fully covered by each reagent. The amount of visible artifact will depend on whether just the edges or the entire section has dried out, in which case the whole section will demonstrate non-specific staining. Wetting agents such as detergents may be added to the rinse buffer, in order to keep the tissue from drying out between and during the staining steps. Detergents also assist with rinsing off unbound antibodies and other reagents, keeping the staining clean. Too much of these reagents can interfere with the staining as well, so controlling the concentration in the rinsing buffer is recommended.

Intrinsic tissue factors

There are two main intrinsic tissue factors that need to be considered as possible causes of non-specific staining.

Endogenous peroxidase is commonly found in red blood cells and other tissue components (Fig. 20.14) and this can react with DAB in horseradish peroxidase detection systems if not treated. This can be blocked by treatment with a 3% hydrogen peroxide solution applied prior to staining to quench the peroxidase activity.

Hydrogen peroxide should be stored in dark bottles and the blocking solution should be freshly prepared before use. Staining may be observed in mast cells in a negative tissue control when inadequate quenching occurs (Fig. 20.15).

Biotin is a vitamin found in high concentration in a number of tissues, including the liver, kidney and brain. This can lead to non-specific staining when using an avidin-biotin detection system, but can be reduced by the addition of a blocking step. To achieve this, avidin is applied first for 15 minutes, rinsed with buffer, and then biotin is applied for 15 minutes, followed by another rinse in buffer. Protein block or the application of the primary follows the avidin-biotin blocking step. Non-specific biotin will be easiest to identify in the negative control slide (Figs 20.16 and 20. 17).

Antibody concentration

All antibodies, whether concentrated or pre-diluted, should have their working dilution validated prior to implementation into the laboratory. Commercially prepared diluents are readily available and generally provide greater stability of diluted

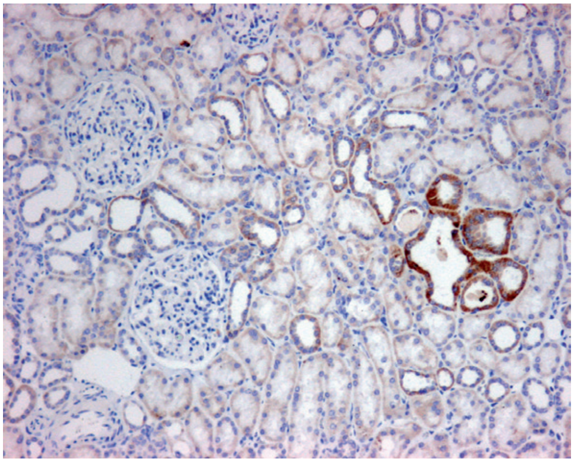

Figure 20.16 Section of kidney intended as a negative reagent control using an avidin-biotin detection system, demonstrating non-specific biotin in some tubules.

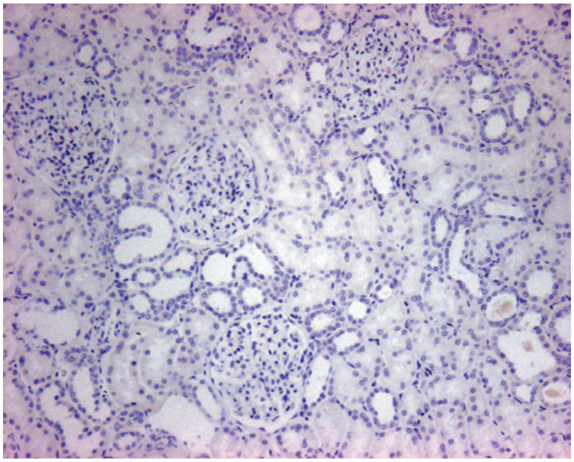

Figure 20.17 Section of kidney (same case as in Fig. 20.16) properly blocked with avidin-biotin, demonstrating the expected absence of staining (negative reagent control).

antibodies. The pH of the diluent is important to maintain the antibody in its proper structure as any deterioration can create non-specific staining and reduce the overall quality of the staining.

The concentration of a pre-diluted ready-to-use antibody has been determined by the manufacturer and may not have been tested with different detection systems. These antibodies should be validated before use by performing serial dilutions (e.g. 1:2, 1:4, 1:8, and so on), starting from the neat concentration, using both positive and negative control tissues. If non-specific staining is observed, the dilution should be taken out further until a good signal-to-noise ratio is achieved.

Polyclonal antibodies are more sensitive than specific. Using monoclonal antibodies reduces non-specific staining, due to their specificity and affinity for one epitope.

Detection system

The choice of detection system used can impact on the quality of staining, but may be affected by other factors such as cost, or reagent rental agreements associated with the type of automated platform being used. In recent years the most commonly used system has been avidin/biotin or streptavidin/biotin, but (as mentioned above) these systems can cause problems, specifically when staining tissue types that contain intrinsic biotin. These are now being replaced by the introduction of polymer or synthetic-based systems that have allowed for the elimination of biotin-induced non-specific staining. Another advantage of the polymer-based systems is that they can shorten staining times by eliminating the need for multiple blocking steps. However, polymer-based systems are not a cure-all for every antibody as some polymeric complexes are large and can have difficulty reaching some epitopes, depending on their location. With the variety of detection systems now available, the laboratory has the opportunity to continually evaluate and improve the quality of the staining produced, provided it can be achieved within the financial constraints of the department.

Chromogen

In general, chromagen solutions should be freshly prepared just before use and according to the manufacturer's instructions, if using a kit. Commercially available products provided for use on automated stainers may be stable for extended periods of time, once mixed in accordance with the manufacturers' instructions. Alkaline phosphatase chromogens are sensitive to light and heat, making them

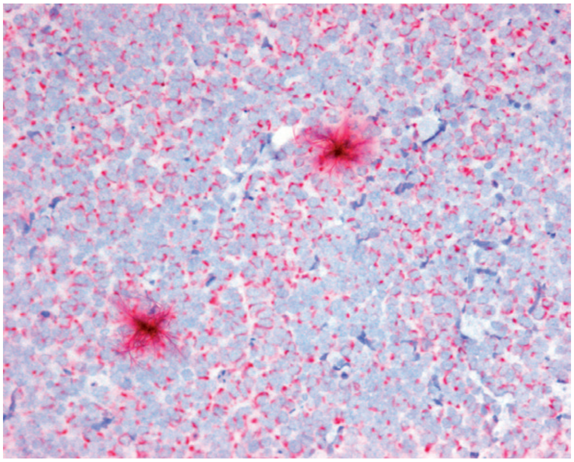

Figure 20.18 Merkel cell tumor stained with an AE1/AE3 cytokeratin antibody cocktail using alkaline phosphatase detection with red chromogen, demonstrating crystals on the surface. Crystal deposits could have been avoided by filtering the chromogen.

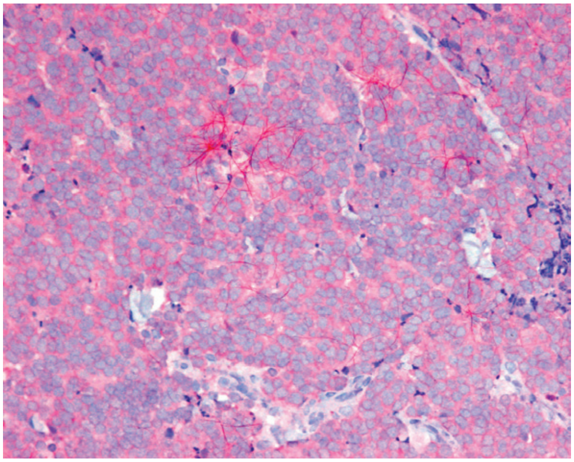

Figure 20.19 Merkel cell tumor stained with an antibody to neuron-specific enolase using alkaline phosphatase detection with red chromogen, demonstrating 'spider web-like' precipitate across the section.

susceptible to lysis/loss after preparation. Peroxidase chromogens can also break down, but not as quickly. Once chromogen activity is depleted, it will either create a blush across the tissue or deposit debris. Chromogen precipitate and streaking can be reduced with adequate mixing, rinsing, and filtering of the chromogen prior to application (Figs 20.18 and 20.19).

Extended time in primary, secondary, or chromogen and inadequate rinsing with buffer between steps may also cause non-specific staining.

Species cross-reactivity

While many commercial kits are available that are ready to use, it is important that the user should understand the formulation of the kit. Those kits that use 'universal' secondary reagents are directed at many primary antibody targets. This information is especially important if staining animal tissue; for example, goat anti-mouse IgG in the kit can cross-react with mouse tissue, creating a non-specific background stain. Mouse tissue typically will demonstrate blood vessel staining due to such cross-reactivity. This staining mimics endothelial cell positive staining. To avoid such non-specific staining, primary antibodies raised in a different species, and secondary reagents not directed against mouse IgG, should be used. If a primary antibody is to be used that originated in the same species being stained, special blocking steps can be introduced to minimize non-specific binding. Kits containing such blocking reagents are commercially available.

Automation error

The use of automated platforms has assisted in improving staining quality and consistency but is not foolproof and can create unique artifacts of its own. Depending on the type of instrument used insufficient rinsing can be a problem – which may be amplified during the chromogen application. Horizontal staining instruments require very careful leveling on installation, to prevent reagents slipping off the slide and causing incomplete coverage of the sections. A faulty probe can result in empty or partial draws of reagents, with the result that it may then dispense air bubbles onto the slide (Fig. 20.20). In instruments that blow over the top of the tissue to mix reagents, the probe height must be set correctly or it can create a bull's eye pattern (Figs 20.21 and 20.22).

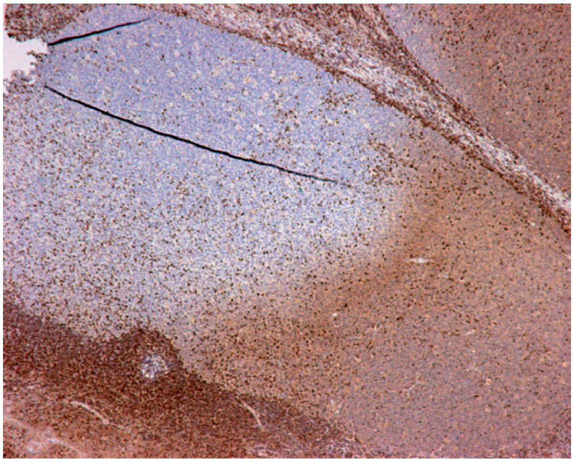

Figure 20.20 Lymph node stained with an antibody to Bcl-2, demonstrating poor tissue coverage due to air bubbles, and non-specific staining due to poor rinsing.

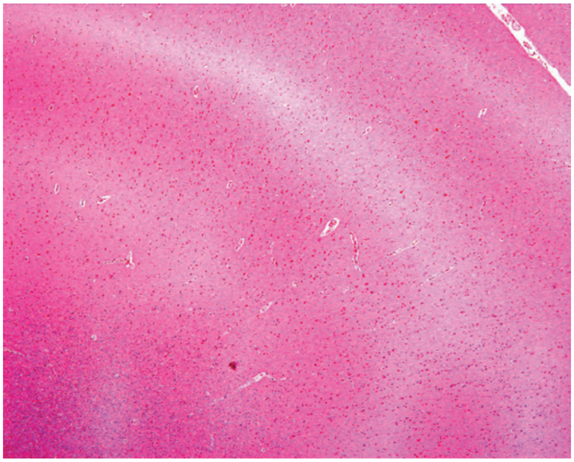

Figure 20.21 Brain stained with an antibody to polyclonal ubiquitin using alkaline phosphatase detection with red chromogen. Bull's eye pattern created on a horizontal stainer with the blowing head too close to tissue.

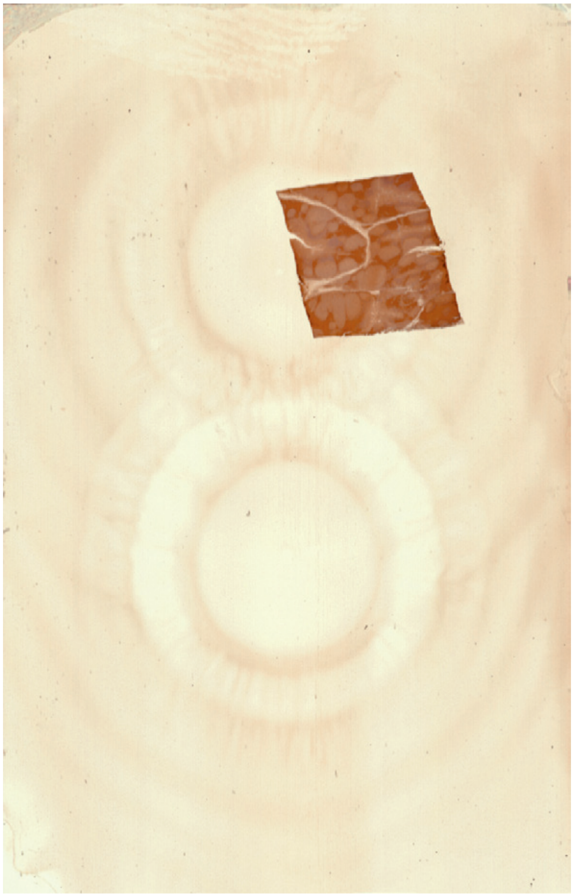

Figure 20.22 Tonsil stained with an antibody to CD3, demonstrating bull's eye artifact on the slide, visible to the naked eye, created by the autostainer's blowing head being too close to the slide.

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